Appendix S1. A survey of post-ejaculatory modifications to sperm (PEMS) throughout the Kingdom Animalia

CONTENTS

I. Cnidaria – Hydrozoa 4

II. Bryozoa – Gymnolaemata 5

III. Mollusca 6

(1) Bivalvia 6

(2) Gastropoda 7

(3) Cephalopoda 9

IV. Annelida – Polychaeta 10

V. Arthropoda 11

(1) Chelicerata 11

(*a*) Acari 11

(*b*) Araneae 14

(2) Crustacea 15

(*a*) Ostracoda 15

(*b*) Copepoda 17

(3) Hexapoda 17

(*a*) Collembola 17

(*b*) Archaeognatha 19

(*c*) Orthoptera 19

(*d*) Blatteria 21

(*e*) Coleoptera 22

(*f*) Hymenoptera 23

(*g*) Lepidoptera 24

(*h*) Diptera 26

(*i*) The fungus gnat (*Sciara coprophila*)

(*ii*) Mosquitoes

(*iii*) The fruit fly (*Drosophila melanogaster*)

VI. Urochordata 31

VII. Craniata 32

(1) Osteichthyes - Teleostei 32

(2) Amphibia 34

(*a*) Anura 34

(*b*) Caudata 35

(3) Testudines 35

(4) Archosauromorpha 37

(5) Mammalia 38

(*a*) Monotremata 38

(*b*) Marsupialia 39

(*c*) Eutheria/Placentalia 40

References 46

A SURVEY OF POST-EJACULATORY MODIFICATIONS TO SPERM (PEMS) THROUGHOUT THE KINGDOM ANIMALIA

Among the taxon-specific PEMS described below, there is tremendous variation in the extent to which systems have been investigated and in the experimental tools employed. Consequently, we have a relatively sophisticated understanding of the cellular and molecular mechanisms underlying PEMS in model systems such as eutherian mammals (i.e. mouse, rat, rabbit and human) and the fruit fly *Drosophila melanogaster*. By contrast, our understanding of PEMS for the majority of taxa is restricted to what can be inferred from ultrastructural comparisons between sperm obtained from the male reproductive tract (MRT) and the female reproductive tract (FRT). In the descriptions below, we attempt to be explicit about methods and to share authors' conclusions and interpretations of their findings. We describe more generalized, taxon-specific aspects of the reproductive biology whenever deemed necessary to understand the described PEMS.

I. CNIDARIA – HYDROZOA

During the sexual phase of their life cycle, the oocytes of hydrozoans develop along a blastostyle within specialized female reproductive polyps called gonangia. Sperm are not capable of entering a gonangium until the oocytes within have matured. To enter, sperm first pass through a funnel-like aperture, then proceed to the surface of an oocyte *via* passageways lined with epithelial cells that lead to and surround each egg (O'Rand, 1974; O'Rand & Miller, 1974). During this journey, sperm undergo two PEMS that are likely (but have not been shown) to be causally linked. First, membrane-bound 'pro-acrosomal' vesicles are progressively lost from the apical and lateral regions of the sperm head as they move through the passageways and

putatively interact with epthelial cells. By the time sperm reach the egg, nearly 90% of vesicles have been lost (O'Rand & Miller, 1974). Second, the sperm become capacitated (i.e. fertilization competent). O'Rand (1972, 1974) experimentally demonstrated the role of epithelial cells surrounding eggs in mediating PEMS. The exposure of egg packets to sperm results in efficient fertilization, but not when eggs are first stripped of their surrounding epithelial cells. Similarly, treatment of intact packets with trypsin prior to the application of sperm also inhibited fertilization, presumably due to the loss of epithelial interaction sites. Interestingly, sperm that had passed through trypsin-treated epithelial cells were incapable of fertilization, yet subsequent exposure of these same sperm to non-trypsin-treated epithelial cells fully restored fertilization capacity.

II. BRYOZOA – GYMNOLAEMATA

The reproductive strategy of the gymnolaemate bryozoan *Membranipora membranacea* is unique among animals in being the only known spermcaster with conjugated sperm (Temkin, 1994; Temkin & Bortolami, 2004). These conjugates are spermatodesms, each containing 64 'sibling' sperm from each cyst that remain associated at the end of spermatogenesis. The sperm of *M. membranacea* may exhibit two different PEMS. First, conjugates dissociate after entering females, after which sperm migrate individually to the surface of ovaries (Temkin & Bortolami, 2004). Second, sperm behaviour is modified after leaving the male and again after contacting a female (Temkin, 1994; Temkin & Bortolami, 2004). Specifically, conjugates exhibit three types of waveforms: small amplitude, large amplitude and reverse (i.e. propagations proceed from the tip to the base of axonemes). The conjugates are motile within the paternal coeloms, become largely quiescent after being spawned, and then recover motility after being drawn into the

lophophores of conspecifics by colony feeding currents. Moreover, the event frequencies and durations of the different waveforms differ between these locations. Experimental evidence suggests that the behavioural changes exhibited by spermatodesms after reaching females are a response to specifically contacting conspecific maternal tissue (Temkin & Bortolami, 2004).

III. MOLLUSCA

(1) Bivalvia

Whereas most species of bivalve molluscs are gonochoristic broadcast spawners with external fertilization, smaller-bodied species within many families exhibit internal fertilization and use the mantle cavity as a brood chamber (Sastry, 1979). Some of these species are self-fertilizing hermaphrodites, but others are spermcasters and have some form of prolonged sperm storage. Clams of the genus *Mysella* brood young in their suprabranchial chambers and store sperm through attachment to specialized regions of the gill lamellae and the gill suspensory membranes (Ó Foighil, 1985*b*). A fine-structure study of sperm transfer and storage in *M. tumida* revealed a PEMS related to gill attachment. The sperm become densely packed with their heads facing the gill filaments. Using light microscopy to examine living gills with attached sperm, Ó Foighil (1985*b*) observed the sperm to be continually buffeted by powerful water currents passing through the gill ostia. Stored sperm were further observed to be actively beating their flagella. Sperm nevertheless remained firmly attached to the gills. Detailed study revealed that attachment is achieved by fine, thread-like extensions of the periacrosomal plasmalemma interdigitating with shorter, stouter gill filament microvilli (see Fig. 2C, D). No fusions of the two types of microvilli were observed but there was close apposition between their respective glycocalices, with postulated glycoprotein crosslinking (Ó Foighil, 1985*b*). The interpretation of the sperm

116 generating head microvilli as an example of PEMS is supported by a fine-structure study of
117 sperm production and release in *M. tumida* (Ó Foighil, 1985*a*). Curiously, sperm in testes have
118 no microvilli on their heads, but do have microvilli similar in appearance emanating from the
119 midpiece. It is not until after they have been packaged into spermatophores and released through
120 the excurrent siphon that the location of microvilli changes (i.e. they disappear from the middle
121 piece and appear on the head; Ó Foighil, 1985*a*,*b*).
122
123 **(2) Gastropoda**
124 All internally fertilizing gastropods that have had their sperm examined before and after
125 insemination appear to share some general features associated with sperm storage and associated
126 PEMS, irrespective of subclass (Prosobranchia, Opisthobranchia, Pulmonata). The descriptions
127 share features in common with that of the polychaete worm *Spirorbis spirorbis* and the bivalve
128 *M. tumida*. The seminal receptacle of prosobranch snails, derived from a differentiated portion of
129 the renal oviduct, is specialized for prolonged sperm storage, with sperm embedding in the
130 epithelial cells (Fretter, 1953; Webber, 1977). A fine-structure study of the gastropod
131 *Cochlostoma montanum* revealed that the inner wall of the seminal receptacle is composed of
132 two cell types, both secretory. Stored spermatozoa insert their heads into invaginations at the
133 apex of both cell types, and develop long slender digitations projecting from the periacrosomal
134 plasma membrane extending towards (but not fusing with) the epithelial cells. The gap is filled
135 with fibrillar material (Giusti & Selmi, 1985).
136 Fine-structure studies of sperm storage in the pulmonate snails *Oxyloma elegans* (Selmi,
137 Bigliardi & Giusti, 1989) and *Arianta arbustorum* (Bojat, Sander & Haase, 2001) similarly found
138 that sperm have their heads embedded within the membranous and microvilli-covered

invaginations of the epithelial cells of the seminal receptacle. However, in contrast with *M. tumida* (Ó Foighil, 1985*b*) and *C. montanum* (Selmi *et al*., 1989), neither pulmonate snail species was observed to develop microvilli/digitations on their sperm heads. Both species did, however, exhibit other PEMS. In *O. elegans*, the mature sperm found in the seminal vesicles of the male portion of the genital duct have the unique feature of a paracrystalline body encircling the apical portion of the nucleus. Sperm that have embedded in epithelial cells of a recipient's seminal receptacle, however, have heads that are slender in appearance and have lost the paracrystalline body (Selmi *et al*., 1989). The sperm of *A. arbustorum* lose their perinuclear sheath within the seminal receptacle and, perhaps as a consequence, the conformation of the acrosome is altered. Sperm in the hermaphrodite duct prior to insemination have an acrosome that is positioned perpendicular to the longitudinal axis of the nucleus, whereas the acrosomal and nuclear axes are nearly confluent for sperm in the seminal receptacle (Bojat *et al*., 2001). This example of PEMS may be triggered by secretions from the epithlial cells, and increased secretory activity of these cells appears to be triggered by the arriving sperm (Bojat *et al*., 2001).

An examination of reciprocal sperm transfer and storage in the opisthobranch *Phyllaplysia taylori* similarly observed sperm with their heads embedded in the epithelial cells of the recepient's seminal receptacle (Beeman, 1972). Although limited evidence is provided, Beeman (1972, 1977) suggests that PEMS in this taxon include substantial modifications to the sperm plasma membrane while in storage.

Some gastropods also exhibit sperm conjugation (see Section IV.2 of main paper), in some cases combined with sperm heteromorphism (for definition and description, see Section V.3*g* of this appendix). In gastropod species with heteromorphic sperm conjugates, the tiny fertilizing eupyrene sperm attach to giant, highly modified oligopyrene sperm, which are hypothesized to

function as ‘mobile penises’ delivering fertilizing sperm to the site of sperm storage within the recipient (Fretter ,1953). After arriving at their destination, the eupyrene sperm must detach from the oligopyrene sperm and then attach to the recipient epithelium.

(3) Cephalopoda

Copulation by cephalopods includes the transfer of spermatophores by a modified tentacle of the male, the hectocotylized third right arm, into the oviduct of the female. After the spermatophores explosively erupt, peristaltic contractions of the oviduct move sperm into the paired oviducal glands (Mann, Martin & Thiersch, 1970; Mann, 1984), where sperm can be stored for months with their heads embedded in the epithelial cells of the spermathecae located within the glands (Froesch & Marthy, 1975). In the octopus, *Octopus vulgaris*, the epithelial cells have cilia and microvilli, yet analysis of fine-structure images suggests these components do not function in sperm attachment. The epithelial cells also lack any preformed invaginations to receive sperm. Rather, it is hypothesized that each sperm ‘drills’ into its host epithelial cell using its screw-shaped acrosome (Froesch & Marthy, 1975; Tosti *et al*., 2001). After embedding, the sperm are inactivated until the time of ovulation (Froesch & Marthy, 1975). PEMS in *O. vulgaris* include the loss of most of the acrosomal membrane that is present in sperm within spermatophores, but absent from sperm in spermathecae. Loss of the membrane exposes the screw-shaped acrosome (see Fig. 3; Tosti *et al*., 2001). Tosti *et al*. (2001) provide experimental evidence that progesterone secreted by the female triggers the PEMS and that sperm from spermatophores possess receptors for progesterone on their plasma membranes.

IV. ANNELIDA – POLYCHAETA

PEMS have been described for two species of marine polychaete worms: *Pisione remota* (family Pisionidae; Alikunhi, 1951; Westheide, 1988) and *Spirorbis spirorbis* (family Serpulidae; Daly & Golding, 1977; Picard, 1980). Despite having extremely different reproductive systems and sperm morphologies, these species share critical aspects of sperm–FRT interactions and in the PEMS exhibited.

Pisione remota has discrete sexes and occupies coarse sand in the shallow intertidal zone. Following direct transfer to females through copulation, the aflagellate sperm are stored within the female's receptacula seminis (Alikunhi, 1951). After arriving in this storage organ, each rod-shaped sperm sheds a vacuole-like structure and the nucleus expands greatly, and the plasma membrane differentiates to grow numerous filliform extensions that envelope apical projections of the females receptacle cells, which were interpreted as relating to long-term sperm storage (Westheide, 1988).

By contrast, *Spirorbis spirorbis* is a simultaneous hermaphrodite that is sedentary and occupies spiral, calcareous tubes. They produce flagellated sperm and are believed to be 'spermcasters' (i.e. sperm that are released into the open water column enter other individuals in the feeding current, followed by internal fertilization; Daly & Golding, 1977). After entering the spermatheca, the sperm heads embed deeply into the spermathecal cells, with each head enclosed by a filiform extension of the spermathecal cell cytoplasm. PEMS involve the development inside the spermatheca of three different membrane specializations for contact with the spermathecal cells: (1) the sperm plasmalemma forms major digitate processes that radiate into the host cell cytoplasm from approximately half-way up the acrosome (see Fig. 2A), (2) minor digitate processes radiate from around the tip of the acrosome, and (3) adjacent to the sperm

nucleus, the membranes of the sperm and spermathecal cell appear thickened by adherence of electron-dense material, with the intracellular space bridged by dense filaments to form scalariform junctions (see Fig. 2A, B; Daly & Golding, 1977; Picard, 1980).

V. ARTHROPODA

(1) Chelicerata

(*a*) *Acari*

The dramatic PEMS of ticks belonging to the family Ixodidae have been the subject of investigation since 1906 (Reger, 1962 and references therein). The sperm of all ticks are aflagellate, yet elongate (up to 1000 μm in some argasid species; Rothschild, 1961) and are motile (Reger, 1974). Motility is attributable to unique cellular processes exposed on the leading tip of the mature sperm and possibly existing as long parallel ridges (Rothschild, 1961; Oliver & Brinton, 1971). Three different kinds of sperm movement have been described: writhing movements restricted to the anterior end, anterior–posterior serpentine contortions and steady gliding moevement (Oliver & Brinton, 1971). Whereas details of the PEMS, sperm storage and the means by which sperm encounter oocytes can differ substantially among taxa, the general process is similar, with sperm being inseminated as 'prospermia' and then undergoing dramatic remodelling, elongation and the activation of motility inside the female within a few hours after insemination (Brinton, Burgdorfer & Oliver, 1974; Mothes & Seitz, 1981).

Fine-structure studies provide detailed descriptions of the process of spermiogensis/PEMS (within both male and female) for the argasid tick, *Amblyomma dissimili* (see Fig. 4; Reger, 1961, 1962, 1963), the ixodid tick, *Dermacentor andersoni* (Brinton *et al*., 1974) and the tetranychid tick, *Tetranychus urticae* (Mothes & Seitz, 1981). The inseminated sperm of

Tetranychus urticae are small, amoeboid cells that lack organelles and a nuclear membrane. They are additionally unusual in posessing a double surrounding membrane: the inner one is the sperm plasma membrane and the outer one is believed to be of somatic origin. Within the receptaculum seminis of the female, the sperm develop what appear to be microtubules beneath the inner membrane. Sperm then increase in size tenfold through the decondensation of chromatin and infiltration of cytoplasmic material, presumptively from the female (Mothes & Seitz, 1981).

The inseminated sperm of *Dermacentor andersoni* are tubular and double in length within the female (Brinton *et al*., 1974), In all cases, it is not a stretch of the imagination to paraphrase the complex process of sperm remodelling within the female of ixodid and argasid ticks as sperm cells turning inside out (see Fig. 4; Shepherd, Levine & Hall, 1982*a*). Oliver & Brinton (1971, p. 734) describe the process as follows: "Capacitation involves sub-terminal rupturing of the peripheral-most membranes at the pointed tip of the elongated spermatid, an extension of the inner core out of the newly open end of the outer sheath with simultaneous sliding of the outer sheath back over the inner core and eventually turning in and contributing to the base of inner core at the opposite end of the cell. Finally, the entire outer sheath turns in and carries the long fusiform nucleus with it. At this stage of development, the cell processes (probably functioning in locomotion) are exposed on the exterior surface of the spermatozoon and extend the length of the cell."

The bizarre PEMS of ticks may relate to the unusual interaction that takes place between sperm and the FRT as part of their unique system of fertilization. Throughout the animal kingdom, there are many instances of sperm binding to or embedding in the epithelium of the FRT (Pitnick *et al*., 2009*b*). However, the only taxa we are aware of in which sperm regularly

penetrate and reside within female epithelial cells (as opposed to embedding in the cell membrane) are in some scale insects (Robison, 1970) and ticks. In both *T. urticae* and *D. andersoni*, sperm are observed to enter epthelial cells lining both the ducts and lumen of the ovaries (Brinton *et al*., 1974; Mothes & Seitz, 1981). Fine-structure observations of *D. andersoni* suggest that penetration of the female epithelial cells by a sperm is critical to its gaining access to the micropyle of an oocyte (Brinton *et al*., 1974).

The extent to which the morphogenesis and activation of tick sperm is male *versus* female mediated is somewhat unclear. The enlargement of *T. urticae* sperm within the female appears dependent on provisioning of cytoplasm from the epithelial cells inhabited by the transforming sperm. By contrast, *in vitro* experiments with sperm of the ixodid tick, *Dermacentor variabilis*, and the argasid, *Ornithodoros moubata*, by Shepherd *et al*. (1982*a*) and Shepherd, Oliver & Hall (1982*b*) have shown that the transformation occurs in two stages: (1) rupture of an operculum at one end of the prospermium and (2) subsequent eversion, elongation and activation. Both stages were successfully triggered and fully executed by exposure of prospermia to polypeptides produced by the male accessory glands that are normally added to the prospermia during ejaculation (Shepherd *et al*., 1982*a*,*b*). Interestingly, exposure of prospermia from either species to accessory gland secretions of the other species failed to trigger the transformation of prospermia (Shepherd *et al*., 1982*b*).

(*b*) *Araneae*

At the end of spermiogenesis within the testes, the sperm of spiders coil and then are encysted within a proteinaceous sheath that is probably secreted by epithelial cells of the deferent duct of the testes. Each small, immotile sphere may contain a single sperm (‘cleistospermia’) or they

277 may be conjugated into various-sized groups ('coenospermia'; Alberti, 1990, 2000; Michalik,
278 Haupt & Alberti, 2004; Michalik, 2007). At the extreme, individual coenospermia of *Liphistius*
279 cf. *phuketensis* can include more than 30 sperm (Michalik, 2007). In addition to sperm, the
280 lumen of the sheath contains other secretory products of the testes or vas deferentia (Michalik,
281 2007). Sperm arrive in the female spermathecae in the encapsulated state and remain so for hours
282 to years depending on the species (Foelix, 2011). A fine-structure analysis of the garden spider,
283 *Argiope bruennichi*, revealed that sperm in the spermathecae are not morphologically different
284 from those in the deferent duct of males (Vöcking, Uhl & Michalik, 2013). The PEMS of spiders
285 thus include decapsulation, uncoiling and activation of sperm motility, with successful PEMS
286 shown to be critical to fertilization (Brown, 1985; Vöcking *et al*., 2013). Vöcking *et al*. (2013)
287 provide evidence that decapsulation and uncoiling of sperm are two morphologically and
288 temporally discrete events, however their ultrastructural analysis could not establish whether
289 sperm activation was synonymous with uncoiling.

290 By varying receipt of sperm relative to female developmental timing (i.e. mated
291 immediately after the final moult *versus* later in adulthood), Brown (1985) experimentally
292 demonstrated with the golden-orb-weaving spider, *Nephila clavipes*, that the timing of PEMS
293 was influenced by the physiological state of the female (i.e. PEMS occurring in approximately
294 18 *versus* 7 days, respectively) and not by maturational differences among males or by the
295 amount of time sperm were stored in the males' pedipalps. Details of sperm–female interactions
296 underlying the PEMS of spiders remain unknown (Herberstein, Schneider & Michalik, 2011).
297 However, the structural complexity of the glandular epithelium of the spermathecae (Uhl, 1994,
298 2000; Michalik *et al*., 2005) and the identification of multiple structural types of secretions
299 associated with sperm within the spermathecae (Vöcking *et al*., 2013) has contributed to a model

300 of females contributing different secretory products at different times, thus providing a "cascade
301 of triggers for decapsulation and uncoiling of sperm" (Herberstein *et al*., 2011, p. 693). It should
302 be kept in mind, however, that male secretions present in seminal fluid have also been observed
303 to be associated with sperm within the spermathecae and may contribute to PEMS (Burger *et al*.,
304 2006; Vöcking *et al*., 2013).
305
306 **(2) Crustacea**
307 (*a*) *Ostracoda*
308 All non-maxillopod crustacea have aflagellate sperm that are either immotile, or their motility is
309 poorly investigated/understood (Jamieson, 1987; Morrow, 2004). Among these, ostracods of the
310 family Cyprididae are unusual in having secondarily evolved filiform, motile sperm. Because
311 such sperm tails derived from an aflagellate ancestor, these tails are aflagellate, with motility
312 accomplished by undulatory waves generated by contractile bands or by unusual
313 membranous organelles (Gupta, 1968; Reger, 1970). The length of sperm varies greatly
314 among species with some having evolved particularly gigantic sperm (range among 51
315 species: 268–11,787 μm; Smith *et al*., 2016).
316 For species examined to date, the sperm of cypridoidean ostracods are immotile in the
317 male and motile within the female seminal receptacle (Matzke-Karasz, Smith & Heb, 2017).
318 Sperm activation in the female has been associated with shedding of a sperm coat inside the
319 seminal receptacle (the female organ is often observed to be filled with empty sperm coats;
320 Wingstrand 1988; Matzke-Karasz *et al*., 2017). However, a recent and rigorous investigation of
321 the ostracod *Mytilocypris mytiloides* reveals that the outer coat ('fibrous coat' of Gupta, 1968;
322 'deciduous coat' of Wingstrand, 1988) of sperm is not moulted; rather, it is composed of

granular material (likely adhered to sperm in the male vas deferens) that is slowly removed during storage in the female. The outer coat is first noticeably less compact after about 5 h in storage, with no traces visible after 15–24 h in storage (Matzke-Karasz *et al*., 2017; also see Gupta, 1968). The timing of dissolution of the outer coat is coincident both with sperm becoming motile and with the initiation of egg fertilization (Matzke-Karasz *et al*., 2017). Matzke-Karasz *et al*. (2017) postulate that the adaptive value of ostracod PEMS may be to (1) adhere adjacent sperm to one another for a more organized insemination and sperm-storage process, (2) suppress motility in the male/activate motility in the female, and/or (3) facilitate the transport and release of bioactive molecules from the male to the female's seminal receptacle. Finally, Matzke-Karasz *et al*. (2017) show that the empty sperm coats frequently observed within females are likely the inner coats of sperm that died and deteriorated within the female.

(*b*) *Copepoda*

Sperm of the copepod *Tisbe holothuriae* are reported to shed their cell coat (i.e. glycocalyx) within the antrum of the female (Pochon-Masson & Garagozlou-van Ginneken, 1978, cited in Ndiaye, Mattei & Thiaw, 1997). However, given the current interpretation of this phenomenon in ostracods (see above; Matzke-Karasz *et al*., 2017), any present interpretation of the mechanism of glycocalyx loss should be considered with caution.

(3) Hexapoda

(*a*) *Collembola*

Collembola are small, soil-dwelling non-insect hexapods with indirect sperm transfer. Males deposit spermatophores in the soil, which females later pick up. By examining sperm from ten

346 species representing eight genera from four families, Dallai *et al*. (2004) identified two unique
347 (and possibly functionally related) attributes of sperm contained within the spermatophores: (1) a
348 central, extracellular cavity containing testicular secretions, and (2) a 'peduncle'. Early in
349 spermiogenesis, a cytoplasmic sleeve forms beneath the sperm head. As flagellar morphogenesis
350 progresses, the sleeve expands to form a large extracellular cavity, the lumen of which
351 progressively fills with a secretion from the epithelial wall of the testis. As the flagellum
352 elongates, it winds to form a lenticular disc surrounding the extracellular mass, all contained
353 within a common plasma membrane. Only those portions of the flagellum extending away from
354 the central cavity are observed to have their own plasma membrane. Once the process of sperm
355 winding is complete, the final steps of sperm maturation within the testes include flattening of
356 the rolled sperm and condensation of the material stored in the central cavity (see Fig. 6).
357 The peduncle is a separate extracellular structure unique to collembolan sperm (Dallai,
358 1970). It is a long, thin, cylindrical structure that adheres to the acrosome and protrudes away
359 from the coiled sperm cell. Length of the peduncle varies among species, but it can exceed the
360 total length of the sperm (see Fig. 6; Fanciulli *et al*., 2017). Similar to the central cavity formed
361 by the coiled sperm, the peduncle is formed late in spermatogenesis by secretions from testis
362 epithelial cells. Biochemical analyses reveal a composition rich in glycoproteins.
363 Sperm cells enter the female spermathecae in a rolled and immotile state with intact
364 peduncles (Dallai *et al*., 2004; Döring, 1986). While stored in the female, the peduncles
365 disappear and the sperm unravel to adopt a filiform shape, thereby releasing the contents of the
366 extracellular cavity into the spermathecae, and they become motile (Dallai *et al*., 2004).
367 Although the triggers for the unique PEMS of collembola are unknown, Dallai *et al*. (2003, p.
368 311) reveal unique specializations of the membrane surrounding the extracellular cavity that they

interpret as “preferential sites for receiving and transmuting environmental signals, especially whatever signal(s) induce these spermatozoa to transform into filiform and motile cells upon reaching the female spermatheca”. Among several alternative hypotheses for the adaptive value of the peduncle, Fanciulli *et al*. (2017) suggest that enzymes released from the peduncle may activate the sperm unrolling process. Based on analyses of the fine structure of the spermathecae and female accessory reproductive glands in one species of collembolan, Dallai, Zizzari & Fianciulli (2008) further postulate that secretions of the spermathecal epithelial cells trigger the dissolution of the peduncle.

(*b*) *Archaeognatha*

For two species of the primitively wingless jumping bristletails, *Machilis distincta* and *Machilinus kleinbergi*, the sperm at insemination have been shown to have an unusual conformation with the flagellum bent like a hairpin within a common plasma membrane (Dallai, 1972). In *M. distincta*, PEMS do not occur until the immotile sperm have reached the spermatheca, at which point the common membrane is lost, essentially doubling the length of the sperm as the flagellum uncoils and becomes motile (see Fig. 7; Dallai, 1972).

(*c*) *Orthoptera*

As with many other insects, the sperm of acridid and catantopid grasshoppers have a thick and somewhat rigid glycocalyx covering the entire cell surface except for the apical acrosome (Longo *et al*., 1993; Lupetti, Mercati & Dallai, 2001). It consists of three layers of glycoprotein and is produced early during spermiogenesis (Baccetti, Rosati & Bigliardi, 1971; Yasuzumi, 1979; Lupetti *et al*., 2001). Post-testicular modifications to the glycocalyx, including elimination

of the outermost layer and some restructuring of the innermost layer begin in the males' seminal vesicles (Lupetti *et al*., 2001). PEMS in these species include the complete dissolution of the glycocalyx within the FRT. Studies of two different species of acridid grasshoppers provide detailed descriptions of the timing and progressive cellular changes that sperm undergo within the female (Renieri & Talluri, 1974; Longo *et al*., 1993). Within the site of insemination (the receptaculum seminis), the glycoprotein caps of bundled sperm dissolve. A couple of hours after insemination, within the female diverticula or along the spermathecal duct, the glycocalyx progressively detaches from the underlying plasma membrane and breaks down; the total process requires 15–24 h. There also may be substantial remodelling of the plasma membrane of sperm (Renieri & Talluri, 1974; Longo *et al*., 1993). Renieri & Talluri (1974) postulate that the dramatic PEMS of acridids are triggered by enzymes secreted by the walls of the spermathecal duct. In support of this hypothesis, Giuffrida & Rosati (1993) were able to trigger glycocalyx dissolution of acridid sperm *in vitro* by incubating sperm collected from male seminal vesicles in extract from female spermathecae [albeit the sequence of events observed and timing of the glycocalyx breakdown differed from *in vivo* observations made by Longo *et al*. (1993)]. Moreover, dissolution of the glycocalyx could only be triggered by spermathecal extract from sexually mature females; sperm were unaffected by extracts from virgin females immediately before or after moulting (Giuffrida & Rosati, 1993).

The PEMS exhibited by tettigoniid orthoptera (katydids) appear different from those of grasshoppers. The sperm of katydids are transferred within spermatophores containing numerous sperm conjugates of variable size (approximately 12–20 sperm per conjugate, depending on species; Viscuso *et al*., 1998). These conjugates have been referred to as 'spermatodesms', but technically are 'bundles' as they are the product of secondary rather than primary developmental

mechanisms (Higginson & Pitnick, 2011). The development of sperm bundles and their ultrastructural features were carefully tracked through the reproductive tracts of both sexes by Viscuso *et al*. (1998, 2002). Four different PEMS have been described. First, within the male and at the time of spermatophore construction, the sperm within bundles have all of their heads embedded in a common mucous-like cap secreted the epithelium of the intratesticular tubule wall. While in the spermatophore, the material forming the cap is disassembled, resulting in the individualization of all sperm. *In vitro* experiments suggest that this process is mediated by products of the male accessory reproductive glands (Viscuso *et al*., 2001). Second, sperm within the female spermathecae reform into bundles that differ structurally from those observed in males. Sperm bundles within females are much larger than those observed in males, with each containing hundreds of sperm. Sperm within female bundles are also much more tightly packed, are linked together by their acrosomes, and exhibit a heightened degree of organization, for example, by the highly ordered, parallel orientation of their acrosomes. Third, relative to sperm in bundles within females, those in males exhibit conspicuously elongated heads containing extra-chromosomal material. Finally, prior to fertilization, PEMS occur in the form of sperm bundle dissociation.

(*d*) *Blatteria*

Sperm of the cockroach *Periplaneta americana* obtained from the female spermathecae were found to exhibit approximately twice the beat frequency of sperm obtained from three locations within the male (testes, seminal vesicles and vasa deferentia) (Hughes & Davey, 1969). Because all sperm were tested under standardized *in vitro* conditions, the difference in beat frequency cannot be attributed to proximate interactions between sperm and FRT morphology, viscosity or

pH. The addition of extracts of either male seminal fluid or female spermathecae to suspensions of sperm from male seminal vesicles had no effect on sperm beat frequency. This result prompted Hughes & Davey (1969) to examine *in vivo* the time course of changes to sperm beat frequency following insemination. Their assay suggests that sperm beat frequency changes occur after approximately 5 h of storage within the FRT. Additional support for these observations constituting PEMS in *P. americana* comes from comparison of the ultrastructure and gross morphology of sperm from seminal vesicles with those from spermathecae. Sperm from the male did not differ from those occupying the FRT for less than 5 h. By contrast, many sperm stored in the female for 10 h and all sperm stored for 48 h exhibited conspicuous modifications to the sperm heads, including compaction of periacrosomal material accompanied by a reduction in the distance between the acrosome and the plasma membrane (Hughes & Davey, 1969).

(*e*) *Coleoptera*

Sperm conjugation is widespread and has evolved to take a diversity of forms among species of diving beetles (Dytiscidae; Higginson *et al*., 2012*a*,*b*, 2015), whirligig beetles (Gyrinidae) (Fig. 1; Breland & Simmons, 1970; Higginson *et al*., 2015) and ground beetles (Carabidae; Sasakawa, 2007; Takami & Sota, 2007; Schubert *et al*., 2017). PEMS in these species obviously include conjugate dissociation (see Section IV.2 of main paper). In both whirligig beetles of the genus *Dineutus* and ground beetles of the tribes Pterostichini, Carabinae and Platynini, an unusual form of primary conjugation has evolved in which all sibling sperm from a single cyst are transferred to females with their heads embedded in a central hyaline rod referred to as a ‘spermatostyle’ (Breland & Simmons, 1970; Sasakawa, 2007; Higginson & Pitnick, 2011; Schubert *et al*., 2017). The conjugates dissociate after reaching the female’s spermatheca, resulting in mated females

461 having what appear to be well-organized stacks of ‘naked’ rods within their spermatheca (S.
462 Pitnick, personal observation).
463
464 (*f*) *Hymenoptera*
465 Some of the most sophisticated research to date on the biochemical environment encountered by
466 insect sperm during insemination (Collins *et al*., 2006; Baer *et al*., 2009*b*), while stored in the
467 female spermathecae (Al-Lawati, Kamp & Bienefeld, 2009; Baer *et al*., 2009*a*) and on
468 associated PEMS (Poland *et al*., 2011) has been performed using the honeybee, *Apis mellifera*.
469 Poland *et al*. (2011) compared the proteome of sperm in ejaculates with that of sperm obtained
470 from the spermathecae of 9–24-month-old queens. Whereas no qualitative differences were
471 found, 15 major sperm proteins exhibited significant differences in abundance between the two
472 samples. Three of these proteins were structural, two were of unknown function, and the
473 remaining ten proteins had functions related to energy metabolism. Consistent with these
474 patterns, enzymatic assays performed for some of these proteins revealed reduced activity by
475 stored relative to ejaculated sperm (Poland *et al*., 2011). It has not yet been possible to
476 discriminate the extent to which the observed changes in sperm during storage in the FRT of *A.*
477 *mellifera* are attributable to sperm ‘senescence’, female-mediated changes or adaptive plasticity.
478 Another group of social hymenoptera, the leaf-cutter ants, have also been shown to exhibit
479 PEMS in the form of changes to sperm motility. Using an *in vivo* assay to investigate sperm–
480 FRT interactions in *Atta colombica*, Liberti, Baer & Boomsma (2016) showed that an extract of
481 the FRT (specifically, the bursa copulatrix and spermatheca) dramatically increases the
482 proportion of motile sperm and sperm velocity relative to sperm exposed to saline or extracts of
483 female haemolymph or hindgut. Interestingly, the same *in vivo* assay applied to another leaf-

cutter ant species, *Acromyrmex echinatior*, revealed that changes to sperm motility are induced by the seminal fluid of rival males, but not by exposure to self seminal fluid (Liberti, Baer & Boomsma, 2018).

(*g*) *Lepidoptera*

With the exception of two species in the genus *Micropterix* (suborder Zeugloptera), males of all species of butterflies and moths exhibit sperm heteromorphism (also referred to as 'sperm polymorphism', 'polymegaly' and 'dichotomous spermatogenesis'), which is the phenomenon of males producing more than one (nearly always two, but see, e.g. Au, Reunov & Wu, 1998) distinct morphological classes of sperm through tightly regulated processes. Sperm heteromorphism has arisen independently numerous times throughout the animal kingdom (and has been the subject of numerous reviews; e.g. Swallow & Wilkinson, 2002; Friedländer, Seth & Reynolds, 2005; Till-Bottraud *et al*., 2005; Higginson & Pitnick, 2011). The different types of sperm perform discrete functions. In virtually all known cases, only one of the sperm types (often referred to as 'eusperm' or 'eupyrene' sperm) ever functions genetically in egg fertilization. The non-fertilizing sperm type (or 'parasperm') may lack a nucleus ('apyrene' sperm), contain only a partial complement of chromosomes ('oligopyrene' sperm), or possess the normal chromosome complement. Irrespective of their nuclear composition, parasperm are not functional in fertilization (e.g. Buckland-Nicks, 1998; Snook & Karr, 1998; Kubo-Irie *et al*., 2003; Hayakawa, 2007). Numerous hypotheses for the adaptive value of parasperm have been proposed (Silberglied, Shepherd & Dickinson, 1984; Swallow & Wilkinson, 2002; Friedländer *et al*., 2005; Till-Bottraud *et al*., 2005; Holman & Snook, 2006), with some receiving empirical support (e.g. Fretter, 1953; Cook & Wedell, 1999; Sahara & Takemura, 2003; Takemura *et al*.,

2006; Holman & Snook, 2008). However, the function of parasperm within the FRT is unknown in most instances.

In Lepidoptera, apyrene sperm comprise 11–99% of the ejaculate, depending on species (Friedländer *et al*., 2005). The two types of sperm differ structurally in other ways. First, the surface of eupyrene sperm are far more complex and 'decorated' than that of apyrene sperm (Friedländer *et al*., 2005). Second, the head cyst cell and the enclosing cyst cells are lysed during release of sperm from the testes only in the case of apyrene sperm, such that all 'sibling' apyrene sperm are individualized and dispersed by the time they reach the seminal vesicle. By contrast, eupyrene spermatozoa remain organized as bundles throughout the male genital duct, with a matrix of 'wavy fibres' binding all sperm together within each bundle. Third, whereas both sperm types are highly modified after leaving the testes, including becoming ensheathed, the characteristics of the outer envelope differ between sperm types. The envelope of individual apyrene sperm, formed by secretions of epithelial cells in the upper vas deferens (Riemann & Giebultowicz, 1991, 1992), is continuous over the entire sperm surface. For eupyrene sperm, the lacinate appendages that uniquely decorate their exterior begin to disintegrate as the cysts leave the testis, with the breakdown products possibly forming the bi-layered envelope that surrounds each spermatozoan (Riemann, 1970; Riemann & Thorson, 1971). Also unique to eupyrene sperm, there is a longitudinal slit in the envelope that is plugged by a reticulate appendage (Friedländer *et al*., 2005).

As far as is known, all lepidopterans exhibit PEMS, including the dissociation of eupyrene bundles within the spermatophore. In *Bombyx mori*, this process has been attributed to the action of initiatorin, a trypsin-like arginine C-endopeptidase secreted by cells in the prostatic region of the male's distal ejaculatory duct, and likely also involves other organic acids, such as succinate,

present in the spermatophore (Osanai, Kasuga & Aigaki, 1989*a*; Aigaki *et al*., 1994; Osanai & Isono, 1997). Dissociation of eupyrene bundles is temporally coupled with the activation of both eupyrene and apyrene sperm motility. Although this appears also to involve the action of cyclic-AMP (Osanai, Kasuga & Aigaki, 1989*b*), the precise mechanisms underlying this phenomenon remain unclear (Friedländer *et al*., 2005). Other PEMS differ between eupyrene and apyrene sperm, consistent with structural differences described above. By the time sperm have reached the female's spermatheca, the eupyrene sperm have lost their reticulate appendage, leaving behind an open slit in the envelope (Riemann, 1970; Riemann & Thorson, 1971). The eupyrene sperm shed their extracellular envelopes in the spermatheca, whereas the apyrene sperm do not (Friedländer, Jeshtadi & Reynolds, 2001). Both the apyrene sperm and any eupyrene sperm that fail to exit from their envelopes eventually degenerate (Friedländer *et al*., 2005).

(*h*) *Diptera*

(*i*) *The fungus gnat,* Sciara coprophila

The sperm of *S. coprophila* are immotile at insemination, during transfer to storage, and throughout their first few hours in the female's paired spermathecae. Then, after approximately 5 h, the sperm become organized parallel to one another, coil and begin to undulate while undergoing dramatic ultrastructural modifications that include (1) elimination from the sperm of nearly all of the electron-transparent, non-paracrystalline component of the mitochondrial derivative, (2) repositioning of the paracrystalline rod and, extraordinarily, (3) the uncoiling and later recoiling of the unusual axial filament complex into a different (see below) configuration (see Fig. 8; Makielski, 1966; Phillips, 1966*a*,*b*). By approximately 7 h after entering the spermathecae, the sperm have uncoiled and the transformation is complete. The sperm, now

553 consistently motile, occupy the periphery of the spermathecal capsules, whereas the sloughed off
554 ribbons of mitochondrial derivative can be observed in knots in the centre of the spermathecae
555 (Makielski, 1966). Phillips (1966*a*) observed that the sloughed mitochondrial material occupies
556 most of the volume of the spermathecae. The axial filament complex of *S. coprophila* sperm is
557 highly unusual, consisting of approximately 70 doublet tubules, each with an associated singlet
558 tubule and organized in a spiral, rather than the 9 + 9 + 2 pattern typical of insect sperm (Phillips,
559 1966*a*,*b*; Dallai, Bernini & Giusti, 1973; Dallai, 2014). The spiral structure lies adjacent to the
560 crystalloid mitochondrial rod near the sperm head, but more caudally and for the entire length of
561 the cell the axial filament wraps completely around the crystalloid (Phillips, 1966*a*,*b*). The
562 enantiomorphic form of the axial filament spiral is the same in all sperm. However, the direction
563 of coiling in all mature sperm from the female spermathecae is invariably the mirror image of
564 sperm from the testes (see Fig. 8; Phillips, 1966*a*,*b*). Makielski (1966) notes that the speed by
565 which the transformation occurs varies among females, requiring about an hour longer in young
566 relative to old females. This observation suggests the transformation is influenced by the female
567 and not strictly attributable to 'programmed' changes intrinsic to sperm, prompting Makielski
568 (1966) to postulate an activational role of the spermathecal fluid.
569
570 (*ii*) *Mosquitoes*
571 Events occurring between insemination and fertilization in mosquitoes, including any known
572 PEMS, were recently reviewed (Degner & Harrington, 2016). The sperm of several species have
573 a thick glycocalyx that is developed in the males' seminal vesicles and is present at insemination,
574 but begins degrading within the spermathecae and is completely removed within 24 h post-
575 mating (Clements & Potter, 1967; Báo & de Souza, 1993; Ndiaye *et al*., 1997). On a perhaps

unrelated note, there have been claims of sperm hyperactivation within the female's bursa copulatrix (Degner & Harrington, 2016), although this is only weakly supported by circumstantial evidence. *In vitro* studies of *Culex quinquefasciatus* show that sperm can exhibit three discrete flagellar wave patterns (Thaler *et al*., 2013), with a similar range of motility having been reported for *Aedes aegypti* (Jones & Wheeler, 1965*a*,*b*). These changes in motility were demonstrated to be calcium-dependent, with the full range of expression triggered *in vitro* by exposure to seminal constituents from the male accessory reproductive organs (Thaler *et al*., 2013). Claims of hyperactivation are based on juxtaposition of the *in vitro* observations of Thaler *et al*. (2013) with the observation that sperm behaviour changes within the FRT. Specifically, rapidly spinning aggregrates have been observed to form in the bursa and at the base of the spermathecal ducts (Degner & Harrington, 2016). These behaviours may be attributable, however, to the effects of viscosity of the FRT, hydrodynamic interactions among sperm, and/or interactions between sperm and FRT architecture.

(*iii*) *The fruit fly, Drosophila melanogaster*

The sperm of *D. melanogaster* undergo molecular changes within the female. One molecular change is the likely fusion of exosomes from the female and/or the seminal fluid with sperm (Corrigan *et al*., 2014). It is not yet known what these exosomes transmit to sperm in *Drosophila* but in mammals, exosomes such as prostasomes and epididymosomes are thought to transport RNAs and proteins into sperm (reviewed in Aalberts, Stout & Stoorvogel, 2014). A second molecular change to sperm is that, at least in *D. melanogaster*, certain seminal proteins bind to the sperm inside the female. Foremost among these is the sex peptide (SP), a 36-amino-acid *Drosophila*-specific seminal peptide. SP causes a multitude of major and important changes to

599 the behaviour and physiology of the mated female fly, including repressing her receptivity to
600 remating and her sleep, and increasing her egg production, feeding, gut size, and aggression
601 (Chapman *et al*., 2003; Lui & Kubli, 2003; Peng *et al*., 2005; Avila *et al*., 2010, 2011; Cognigni,
602 Bailey & Miguel-Aliaga, 2011; Apger-McGlaughon & Wolfner, 2013; Bath *et al*., 2017). Some
603 of these effects are mediated through the female's receptor for SP, a G-protein coupled receptor
604 called sex peptide receptor (SPR) (Yapici *et al*., 2008). Interestingly, these post-mating changes
605 have (where tested) been shown to require the presence of sperm and persist while the female is
606 storing sperm (Manning, 1967; Kalb, DiBenedetto & Wolfner, 1993). This led Eric Kubli and
607 colleagues to hypothesize, and then demonstrate, that SP binds to sperm (*via* its N-terminal
608 region) and is thus retained within the female together with sperm. SP is bound along the entire
609 length of the sperm, suggesting that sperm might serve as a protected storage site for SP (Peng *et*
610 *al*., 2005). SP does not bind to sperm on its own, but requires a network of approximately nine
611 other seminal proteins (proteases, lectins, cysteine-rich secreted proteins) to mediate its binding
612 (Ravi Ram & Wolfner, 2009; LaFlamme, Ravi Ram & Wolfner, 2012; Findlay *et al*., 2014;
613 Singh *et al*., 2018). SP and the network proteins are found bound to sperm soon after entry into
614 the female, and they enter the storage organs with the sperm. Within 1–2 days the network
615 proteins have disappeared, presumably having modified the sperm, SP, or both to allow SP to
616 remain bound to sperm. Over time, the C-terminal (active) portion of SP is gradually cleaved
617 from sperm (Peng *et al*., 2005) by a trypsin-family protease of as-yet unknown origin.
618 Interestingly, this cleavage occurs primarily on SP bound to the sperm's tail (Peng *et al*., 2005),
619 suggesting that there may be molecular differences in the nature of SP binding to the tail *versus*
620 the head of sperm, but these are unknown. The released C-terminal peptide can then bind to SPR

621 and cause continued post-mating changes in receptivity, egg-laying, and other phenomena, in the
622 female (Yapici *et al*., 2008).

623 *Drosophila melanogaster* sperm may undergo additional PEMS that modulate their motility.
624 Support for this contention comes from two reports that focus on effects of the sperm-enriched
625 protein Amo, which is a homolog of the human transient receptor potential channel TRPP2,
626 encoded by the gene *PKD2* (Gao, Ruden & Lu, 2003; Watnick *et al*., 2003). Amo is localized at
627 sperm heads and the tips of flagella, and *D. melanogaster* males with Amo mutations produce
628 and ejaculate motile sperm that fail to reach or to be retained in the female sperm-storage organs
629 (Gao *et al*., 2003; Watnick *et al*., 2003; Köttgen *et al*., 2011). First, the waveform of wild-type *D.*
630 *melanogaster* sperm was reported to differ when they are in the bursa *versus* in the seminal
631 receptacle (Yang & Lu, 2011), with abnormal waveforms seen in sperm lacking *PKD2*. Second,
632 in a single investigation using transgenic *D. melanogaster* with distinct fluorescent tags on their
633 sperm heads and flagellae, Köttgen *et al*. (2011) quantified sperm beat frequency *in vitro*. They
634 reported that although sperm released into saline from the male's seminal vesicles of wild-type
635 and Amo-mutant males did not differ in beat frequency, wild-type sperm released from the
636 mated female's bursa exhibited a significantly faster beat frequency than did sperm from Amo-
637 mutant males, suggesting a change within the FRT that depended on the presence of Amo in
638 sperm.

639 Other PEMS may occur in some *Drosophila* species with giant sperm. Variation in sperm
640 length among species of *Drosophila* exceeds that in the remainder of the animal kingdom
641 (Pitnick, Markow & Spicer, 1995*a*). For some species, such as *D. bifurca* with 58,290 μm long
642 sperm (Pitnick, Spicer & Markow, 1995*b*; Lüpold *et al*., 2016), the sperm are individually rolled
643 into balls within the MRT (Joly, Luck & Dejonghe, 2008). Shortly after insemination, the sperm

balls unravel as they enter the female's approximately 8 cm long seminal receptacle (Pitnick, Markow & Spicer, 1999). We are unaware of studies of the molecular basis for this dramatic conformational change, but we think it is likely to involve PEMS.

VI. UROCHORDATA

Tunicates exhibit a diversity of asexual and sexual reproductive systems. The colonial ascidian, *Diplosoma listerianum*, is an outcrossing hermaphrodite. They are spermcasters with sperm released from the seminal vesicles of individual zooids that leave the colony through a common exhalent opening to disperse into the open sea. Sperm enter the fertilization canals of acting female zooids. Fertilization is internal, within a cloacal chamber that is isolated from sea water (Burighel & Martinucci, 1994*a*,*b*). Sperm reach the chamber containing eggs by passing down a 'fertilization canal' formed by a hollow, single-cell thick, tube-like extension of the ovary. At the base of this canal, sperm must pass through the ovary epithelium by using their corkscrew-shaped heads to spiral through the intercellular junctions binding adjacent epithelial cells (Burighel & Martinucci, 1994*b*). Once in this storage location, sperm may remain viable for up to one month (Bishop & Ryland, 1991).

Sperm–female interactions in *D. listerianum* have been shown to be intimate and sophisticated, with epthelium of the fertilization duct being capable of 'assessing' sperm surface proteins and blocking passage by genetically incompatible (e.g. self) sperm to the cloacal chamber. Those sperm sharing self-recognition markers with the maternal tissue are removed *via* immune-like phagocytotic processes (Burighel & Martinucci, 1994*a*; Bishop, 1996; Bishop, Jones & Noble, 1996). Remarkably, this system has also been shown to favour sperm of

genotypes that are under-represented in the population (a ‘rare male effect’; Pemberton, Noble & Bishop, 2003).

The fertilization canal of *D. listerianum* is also the site of unique PEMS. All sperm from the testes or those entering the fertilization duct have an unusual head structure characterized by having the nucleus flanked by both a unique elongate mitochondrion and by endoplasmic derivatives. The head also possesses a dense groove, which is an invagination of the plasmalemma bound to the nuclear envelope and which runs spirally around the entire head (see Fig. 10; Burighel, Martinucci & Magri, 1985). In striking contrast, sperm that have successfully traversed the fertilization canal have their mitochondrion and endoplasmic derivatives restricted to the posterior half of the head, with the anterior portion having become much thinner and more needle-like. The dense groove has detached from the base of the head and transformed into a helical whorl that wraps around the head seven or eight times, giving the anterior portion of the head the appearance of a corkscrew (see Fig. 10; Burighel & Martinucci, 1994*a*).

Some of the cellular machinery underlying the unusual PEMS of *D. listerianum* may be homologous with the ‘sperm reaction’ of solitary ascidians with external fertilization. Upon contact with the vitelline coat of an egg, the mitochondrion is rapidly translocated from the head to the tail, from which it is then lost completely (Ursprung & Schabtach, 1965; Lambert, 1982).

VII. CRANIATA

(1) Osteichthyes – Teleostei

The majority of bony fish species reproduce by spawning, during which paired females and males coordinate their simultaneous release of gametes. Fertilization happens rapidly, and sperm tend to be short-lived relative to those of externally fertilizing species with broadcast release of

689 gametes (Johnson & Yund, 2004; Browne et al., 2015). Fish sperm are typically activated upon
690 contact with water (see Section IV.1 of main paper). Their behaviour is subsequently modified
691 by contact with ovarian fluid (coelomic fluid), a semiviscous liquid derived from the ovarian
692 secretory epithelia. This material contains proteins, sugars, hormones and inorganic ions and is
693 released by females with eggs and retained in a boundary layer around each egg (Lahnsteiner,
694 Weismann & Patzner, 1995; Rosengrave *et al*., 2009; Johnson *et al*., 2014). Ovarian fluid acts as
695 a chemoattractant for sperm and influences sperm performance, including increases to the per
696 cent motile sperm, swimming speed, linearity of movement, longevity, wave amplitude and
697 swimming efficiency, with variables generally increasing with the concentration of ovarian fluid
698 (e.g. Turner & Montgomerie, 2002; Rosengrave *et al*., 2009; Butts *et al*., 2017).
699 Internal fertilization has arisen independently at least eight times in fish (Stockley *et al*.,
700 1997), thus setting the stage for enhanced PEMS mediated by sperm–female interactions in those
701 taxa. Even with internal fertilization, eggs within the female are still bathed in ovarian fluid. In
702 the guppy, *Poecilia reticulata*, males transfer sperm to females along a groove in the
703 gonopodium, a fin that has been modified to function as an intromittent organ. Perhaps as an
704 adaptation to minimize sperm loss during transfer, the sperm are packaged as bundles of variable
705 size that later undergo PEMS in the form of dissociation. Gasparini & Pilastro (2011) identified
706 possible additional PEMS in this species. First, using artificial insemination, they showed that
707 unrelated males have a competitive fertilization advantage over full siblings of females. Next,
708 using *in vitro*, computer-assisted semen analysis, they provide compelling evidence that the
709 fertilization advantage of unrelated males is attributable to ovarian fluid-mediated PEMS.
710 Specifically, sperm velocity, a known predictor of competitive fertilization success in this

711 species, was significantly influenced by relatedness, with sperm swimming more slowly in the
712 presence of ovarian fluid from a sibling female.
713
714 **(2) Amphibia**
715 (*a*) *Anura*
716 The majority of frog and toad species reproduce by external fertilization. While a mating pair is
717 in amplexus, the female releases strands of eggs within a jelly matrix and the male ejaculates
718 onto the jelly. The jelly envelopes surrounding the eggs are deposited in 5–8 (depending on
719 species) distinct morphological layers as the eggs pass through different regions of the oviduct
720 (Shivers & James, 1970*b*; Jégo, Joly & Boisseau, 1980; Coppin, Maes & Strecker, 2002).
721 Investigations of the histochemical properties of the the jelly glands across the oviduct and of the
722 corresponding jelly layers in numerous species of frogs and toads has revealed that the jelly is
723 mainly composed of glycoproteins of a mucin type that contains up to 50% carbohydrates (e.g.
724 Shivers & James, 1970*b*; Coppin *et al*., 2002). Interestingly, the carbohydrate content exhibits
725 striking species specificity (Coppin *et al*., 2002, and references therein).
726 The requirement of the jelly coat for fertilization was first established by Newport (1851). A
727 critical role for the jelly in frog PEMS is now clearly established. Experiments with the frog,
728 *Rana pipiens*, utilizing a fully factorial design to compare fertiization rates of oocytes with or
729 without jelly when exposed to sperm that either had or had not been previously exposed to jelly,
730 were the first clear demonstration that the jelly capacitates sperm (Shivers & James, 1970*a*). This
731 conclusion was reinforced by a study showing that treatment of *R. pipiens* jelly-coated eggs with
732 an antibody to jelly prevents their fertilization whereas antibody-treated eggs are successfully
733 fertilized by sperm that were previously capacitated by exposure to untreated jelly (Shivers &

James, 1971). By exposing sperm of the toad *Rhinella (Bufo) arenarum* to diffusible substances from the jelly coat (referred to as "egg water"), Krapf *et al*. (2007) showed that sperm attaining a capacitated state was correlated with a loss in sperm cholesterol and an increase in protein phosphorylation of tyrosine residues. Exposure to egg water was further shown to be necessary for the acrosome reaction to occur. In the absence of jelly, sperm still bind to the vitelline envelope, but acrosomal exocytosis does not occur (Krapf *et al*., 2009). Finally, diffusible factors from the egg jelly have been shown to regulate sperm motility. Toad and frog sperm are activated by exposure to the lower osmolarity of the fertilization environment (i.e. pond water). However, such exposure only initiates *in situ* movement. The transition to progressive movement requires exposure to egg jelly or egg water (Simmons, Roberts & Dziminski, 2009; Krapf *et al*., 2014).

(*b*) *Caudata*

To date, the described PEMS of salamanders and newts are restricted to the activation of sperm motility. Most members of this order reproduce by internal fertilization, with sperm transferred to females in spermatophores and then stored quiescently within spermathecae (Wake & Dickie, 1998; Sever, 2002). The eggs are coated with discrete, concentric layers of jelly sequentially deposited along the oviduct as in externally fertilizing amphibians. Fertilization occurs within the female's cloaca, with sperm motility activated upon contact with the jelly. An investigation of fertilization in the newt *Cynops pyrrhogaster* revealed the presence of six layers of jelly, each with some unique carbohydrate components (Watanabe & Onitake, 2002). The outermost layer contains both a sperm motility-initiating substance (SMIS; likely a 34 kDa protein) and an associated acrosome reaction-inducing substance (ARIS) (Watanabe *et al*., 2003, 2010).

(3) Testudines

In the Chinese soft-shelled turtle, *Pelodiscus sinensis*, spermatogenesis is seasonal and, following spermiation, sperm spend many months within the male epididymis and then the female oviduct (Zhang *et al*., 2008). By comparing the fine structure of sperm obtained from these two locations, Zhang *et al*. (2015) detail substantial modifications to sperm over this protracted time period. Sperm found in the epididymis have a large cytoplasmic droplet attached along the posterior end of the head and flanking the entire midpiece. These cytoplasmic droplets change over time, with each containing, on average, 7.0 lipid droplets and 4.1 vacuoles in the late autumn and 1.4 lipid droplets and 14.5 vacuoles in the late spring. By contrast, sperm obtained from the oviduct or from the females' sperm-storage tubules (SSTs) had no associated cytoplasmic droplets (see Fig. 11). The authors interpreted the contents of the cytoplasmic droplet as an endogenous source of energy for sperm in the epididymis (Zhang *et al*., 2015). The structure of the mitochondria in the midpiece also differed across locations and over time. Sperm from the epididymis and those stored for only a short time in the oviduct had mitochondria exhibiting an onion-like ultrastructure with 8–15 concentric laminated membranes circling a dense substrate core. At more intermediate stages of sperm storage in the oviduct, the number of membrane layers decreased, gaps appeared between the layers, and the volume of mitochondria was reduced, resulting in a thinning of the midpiece. The midpiece thinned further still later in the season as a result of the mitochondria returning to their normal structure of a double membrane with cristae and tight packing (see Fig. 11). These modifications were interpreted as sperm transitioning to mitochondrial metabolism, allowing ATP production *via* the oxidative phosphorylation (OXPHOS) pathway prior to fertilization (Zhang *et al*., 2015). Note that a study

780 of the painted turtle, *Chrysemys picta*, similarly observed a large cytoplasmic droplet containing
781 lipid droplets associated with the midpiece of epididymal sperm, with the droplet disappearing
782 just prior to mating and being absent from oviductal sperm (Gist *et al*., 2002).
783
784 **(4) Archosauromorpha**
785 Post-testicular sperm maturation, including the acquisition of fertilization capacity in the FRT
786 (i.e. capacitation; see below) is a hallmark of eutherian mammal sperm. Recent investigations in
787 the Australian saltwater crocodile, *Crocodylus porosus*, using mammalian *in vitro* capacitation
788 conditions, supports analogous capacitation processes in the Crocodilia (Nixon *et al*., 2016*a*,
789 2019*b*). This includes the induction of sustained motility, activation of cyclic AMP (cAMP)
790 signalling pathways and protein phosphorylation mediated by protein kinase A (PKA). Use of
791 comparative and quantitative proteomics revealed 126 proteins that were differentially
792 phosphorylated in capacitated *versus* non-capacitated sperm. These same proteins exhibited
793 substantial evolutionary overlap with those implicated in mammalian sperm capacitation, and
794 included elements of metabolic, signal transduction and cellular remodelling pathways (Nixon *et*
795 *al*., 2019*b*).
796 By contrast, experiments utilizing *in vitro* fertilization with the fowl, *Gallus domesticus*,
797 turkey, *Meleagris gallopavo*, and Japanese quail, *Coturnix coturnix japonica*, indicate
798 spermatozoa of these species do not require a period of capacitation within the female in order to
799 fertilize an oocyte (Howarth, 1971; Howarth & Palmer, 1972; Nixon *et al*., 2014). Indeed, the
800 sperm of fowl and quail show a rapid acrosome reaction *in vitro* in the presence of an oocyte's
801 perivitelline membrane (Horrocks *et al*., 2000; Nixon *et al*., 2014). Nevertheless, a recent study
802 of *G. domesticus* identified putative PEMS in the form of small vesicles (microvillus blebs,

MvBs, released from the apical tips of epithelial cells by apocrine secretion) fusing to the plasmalemma of sperm within the sperm-storage tubules of females. Several hypotheses for the function of this PEMS were suggested, including the reversible suppression of premature capacitation and stabilization of the plasmalemma (Bakst & Bauchan, 2015).

(5) Mammalia

(*a*) *Monotremata*

Sperm of the short-beaked echidna, *Tachyglossus aculeatus*, and of the platypus, *Ornithorhynchus anatinus*, have been shown to conjugate into relatively large bundles (typically about 100 spermatozoa per bundle) during passage through the epididymis (Djakiew & Jones, 1983; Johnston *et al*., 2007; Nixon *et al*., 2011, 2016*b*). These bundles obviously must dissociate within the FRT (see Section IV.2 of main paper). Whereas the adaptive value of sperm conjugation is unknown in these species, one of several hypotheses put forward by Johnston *et al*. (2007) was that conjugation is a mechanism for limiting the premature capacitation of sperm. Whether or not monotreme sperm capacitate (in addition to merely disassociating from bundles), however, is an open question. *In vitro* studies with echidna and platypus sperm suggest that the molecular process underlying any sperm capacitation must differ from that of eutherians, in that the elevation of intracellular cAMP levels fails to increase protein phosphorylation. Thus, either cAMP acts through alternative pathways or eutherian-like capacitation does not occur in monotremes (Nixon *et al*., 2016*b*).

826 (*b*) *Marsupialia*

827 The sperm of some Australian marsupials have been shown to undergo PEMS within the FRT,
828 but the processes and their regulation clearly differ from those involved in capacitation in
829 eutherian mammals, and even from New World marsupials (Bedford, 1996; Mate & Rodger,
830 1996). Due to these differences, the first successful *in vitro* fertilization (IVF) with a marsupial,
831 in this case with the South American grey short-tailed opossum, *Monodelphis domestica*, did not
832 occur until 1993 (Moore & Taggart, 1993), and traditional IVF has never been achieved with an
833 Australian marsupial, although fertilization through intra-cytoplasmic sperm injection has been
834 accomplished (Mate *et al*., 2000; Magarey & Mate, 2003; Richings *et al*., 2004).

835 The capacity to fertilize an oocyte appears to be achieved by *M. domestica* sperm simply by
836 the dissociation of the paired sperm, which can be induced *in vitro*, and may not involve the
837 same capacitation mechanisms described for eutherian mammals (see below; Moore & Taggart,
838 1993). Although the ability to fertilize was not tested, paired sperm of the Virginia opossum,
839 *Didelphis virginiana*, unpair under similar culture conditions *in vitro*, and their unpairing *in vivo*
840 within the oviduct just prior to fertilization has also been documented (Rodger & Bedford, 1982).

841 The acrosome of Australian marsupials has been described as "remarkably stable" and the
842 acrosome reaction is not inducible by reagents effective for eutherian sperm (Sistina *et al*.,
843 1993*a*,*b*; Rodger, 1994; Bedford, 1996; Czarny, Mate & Rodger, 2008). Whereas fertilization in
844 eutherian mammals entails fusion of the egg membrane with the central equatorial segment of
845 the sperm head, the sperm of Australian marsupials lack an equatorial segment (Rodger, 1994).

846 Interestingly, the marsupial sperm head shifts, relative to the tail, between a linear and a 'T'
847 conformation, with the latter involved in sperm–egg binding in *Sminthopsis crassicaudata*

(Bedford & Breed, 1994; see Fig. 12). Sperm appear to complete spermatogenesis in the T confirmation, subsequently transition to the linear morphology during epididymal maturation, and then adopt the T conformation again in the FRT (Setiadi, Lin & Rodger, 1997; Lin & Rodger, 1999; Mate *et al*., 2000). Induction of the T conformation has also been linked to sperm–oviduct epithelium interactions in the brushtail possum, *Trichosurus velpecula* (Sidhu *et al*., 1999*a*), and the Bennett's wallaby, *Macropus rufogriseus rufogriseus* (Boere, Diaz & Holt, 2011). Although it has been suggested that T-conformation acquisition may directly preceed capacitation (Mate & Rodger, 1996), clear links to sperm functionality have yet to be established. Although evidence is limited, it has also been claimed that FRT secretory proteins bind to sperm and enhance *in vitro* survival and motility in the brushtail possum (Sidhu *et al*., 1999*b*), although further investigation is required.

(*c*) *Eutheria/Placentalia*

All eutherian sperm are believed to exhibit PEMS in the form of capacitation, although it is becoming apparent that capacitation may exhibit species-specific characteristics and is therefore a process likely to include a diversifying suite of PEMS. The term 'capacitation' originally referred to the set of requisite changes sperm must undergo after prolonged residence in the FRT to acquire fertilization capacity (Austin, 1951, 1952; Chang, 1951). These observations were based on *in vivo* experiments in non-human mammals and, at the time, the underlying mechanisms were unknown. Despite the fact that human *in vitro* fertilization efforts pre-date the original use of the term capacitation (Pincus & Enzmann, 1934), human sperm capacitation was not successfully induced *in vitro* until 1969 (Edwards, Bavister & Steptoe, 1969), rapidly followed by the Nobel award winning research that achieved human *in vitro* fertilization

871 (Edwards, Steptoe & Purdy, 1970). Due to the difficulty of studying capacitation *in vivo*, sperm
872 capacitation has been and continues to be studied largely *in vitro* and our understanding of the
873 FRT environment with regard to capacitation-related PEMS has advanced relatively little (see de
874 Jonge, 2017, for an excellent historical perspective).

875 Prior to providing an overview of the PEMS involved in mammalian sperm capacitation, it
876 should be noted that the meaning of the term 'capacitation', even when applied strictly to
877 mammals, has been debated. Capacitation encompasses a diverse array of PEMS that
878 traditionally included two primary processes: (1) hyperactivation, which is the transition to high-
879 amplitude asymmetric flagellar beating, and (2) the acrosome reaction, which is the exocytotic
880 event exposing the acrosomal contents, including the oocyte cumulus matrix penetrating enzyme
881 hyaluronidase and molecules participating in gamete fusion (Chang, 1984). Others, however,
882 excluded the acrosome reaction and defined capacitation as the processes required to prepare
883 sperm to undergo the acrosome reaction (Bedford, 1970; Florman & Babcock, 1991). The factor
884 distinguishing these perspectives is whether the acrosome reaction is induced during the final
885 stages of sperm migration or whether acrosome-intact sperm pass through the oocyte cumulus
886 matrix and experience an interaction with zona pellucida proteins that initiate the acrosome
887 reaction (reviewed by Florman & Fissore, 2015). We adhere to the traditional view regarding the
888 acrosome reaction as the termination of capacitation and include an overview of recent acrosome
889 reaction studies in our discussion below. Lastly, the importance of *in vitro* studies in model
890 organisms to our understanding of capacitation cannot be overstated, although the use of these
891 systems as surrogates for *in vivo* studies of capacitation across mammals has recently come
892 under scrutiny (Okabe, 2014; Kaupp & Strunker, 2016). As such, our overview of capacitation is

893 reliant on generalizations derived from model organism studies, although we highlight species-
894 specific aspects of capacitation PEMS where they have been studied.

895 The critical challenge facing sperm biologists is linking the well-established biochemical
896 transitions and signalling events of capacitation to the *in vivo* life history of sperm in a highly
897 selective FRT environment. Of the hundreds of millions of sperm inseminated as part of a typical
898 mammalian ejaculate, only a small number are estimated to arrive successfully at the surface of
899 the egg (Williams *et al.,* 1992; Suarez & Pacey, 2006). Sperm begin this journey with highly
900 progressive motility and migrate through the cervical mucus, which is believed to biochemically
901 alter sperm and initiate the remodelling and removal of membrane sterols (cholesterol and
902 desmosterol) (de Jonge, 2017). Sperm are also exposed to reactive oxygen species (ROS)
903 produced by leukocytes, which promotes the initiation of critical capacitation signalling
904 pathways (see below) and, importantly, are believed to inhibit the progression of immature or
905 low-quality sperm (de Jonge, 2017). Sperm migration is then actively assisted by contractions in
906 the uterus, and upon reaching the oviduct, progressive motility ceases and sperm form a close
907 association with the epithelium of the isthmic region (termed the 'sperm reservoir'; Suarez,
908 2002; Suarez & Pacey, 2006). Residence in the sperm reservoir provides the opportunity for
909 capacitation to be temporally linked to ovulation, when biochemical changes (such as changes in
910 pH) promote hyperactivation and progression to the site of fertilization (Suarez, 2008). It is
911 currently unclear whether achieving hyperactivation is sufficient for release from the sperm
912 reservoir or whether molecular changes in sperm–epithelium binding affinity play an integral
913 role (reviewed in Suarez, 2016). It is suspected that changes in binding affinity could be induced
914 by the effects of oviductal epithelial secretions on sperm and that the specific molecular
915 interactions underlying binding could be species specific (Coy *et al*., 2012). If empirical

evidence supporting this phenomenon become available, the dynamics of sperm release from the sperm reservoir would be a PEMS-dependent process linked to novel sperm × female molecular interactions.

The ability to mimic *in vivo* capacitation under *in vitro* conditions, in conjunction with the use of genetic tools, has been fundamental to characterizing the biochemical reactions and signalling pathways governing capacitation [see Gervasi & Visconti (2016) and Aitken & Nixon (2013) for excellent overviews]. The critical molecular event in sperm capacitation is the activation of cAMP pathways, which ultimately result in increased intracellular Ca^{2+} levels necessary for the induction of hyperactivation. The activation of cAMP occurs through the synergistic activating effects of HCO_3^- (Gadella & Visconti, 2006) and reactive oxygen species (ROS) on soluble adenylate cyclase, which converts ATP into the second messenger cAMP. Enhanced cAMP levels, in turn, activate PKA and inhibit serine/threonine phosphatases. These pathways, in conjunction with ROS-induced efflux of sterols from the sperm plasma membrane, alter sperm membrane protein and lipid composition in the sperm head (Harrison & Gadella, 2005). This membrane hyperpolarization, accompanied by cAMP-mediated increases in pH, is believed to be conserved across mammals and essential to the activation of sperm ion exchangers that modulate sperm membrane potential and intracellular increases in Ca^{2+} levels. In turn, release of Ca^{2+} from stores located at the base of the sperm head induce the stereotypical transition in hyperactive flagellum wave form (Ho & Suarez, 2003). The importance of Ca^{2+} for the initiation of mammalian hyperactivation, and ultimately the acrosome reaction, has been long recognized (Yanagimachi, 1982), and the molecular basis of this relationship is being successfully elucidated using genetic approaches (reviewed by Gervasi & Visconti, 2016).

938 The traditional view of the acrosome reaction postulated that this exocytotic event was
939 induced through interactions with the zona pellucida, and more specifically zona protein 3 (Zp3),
940 after successfully traversing the oocyte cumulus matrix (Saling & Storey, 1979; Florman &
941 Storey, 1982; Bleil & Wassarman, 1983). However, the ability to track mouse sperm fate using
942 transgenic green fluoresecent acrosome tags (Nakanishi *et al*., 1999) has led to the unexpected
943 observation that most fertilizing sperm undergo the acrosome reaction prior to interactions with
944 the zona (Jin *et al*., 2011) and that acrosome-reacted sperm have the capacity to fertilize oocytes
945 with intact zona (Inoue *et al*., 2011). Consistent with this biology is the observation that
946 acrosome-intact sperm are found in the isthmus of the oviduct but sperm in the upper isthmus
947 and ampulla are acrosome-reacted (La Spina *et al*., 2016; Muro *et al*., 2016). These findings
948 support the traditionally held view that the acrosome reaction is part of capacitation. However, it
949 is noteworthy that sperm that have penetrated the zona pellucida, but have not fused with the
950 oolema, remain vigorously motile and fertilization competent (Kuzan, Fleming & Seidel, 1984;
951 Inoue *et al*., 2011). Nonetheless, the acrosome reaction is critical in that it results in the exposure
952 and translocation of sperm proteins participating in sperm–oocyte interactions, including Izumo
953 1, which is required for sperm–egg fusion (Okabe *et al*., 1987; Miranda *et al*., 2009; Aydin *et al*.,
954 2016; Ohto *et al*., 2016).
955 In addition to capacitation, other PEMS have been described for eutherian mammals. For
956 example, bovine seminal vesicle proteins (BSPs) bind to sperm and mediate the formation of the
957 sperm reservoir through interactions with the oviductal epithelium (Hung & Suarez, 2012).
958 Under *in vitro* capacitating conditions, some of these proteins are either lost from sperm or
959 cleaved. It remains unclear whether these changes occur *in vivo* or whether they are induced by
960 the FRT. Several species of eutherian mammals also produce sperm conjugates that must

961 dissociate within the FRT. Examples include the sperm ‘trains’ and ‘rosettes’ of several muroid
962 rodent species (Immler *et al*., 2007; Monclus & Fornes, 2016) and the ‘rouleaux’ of some species
963 of guinea pig, armadillo, squirrel and loris (Higginson & Pitnick, 2011; Monclus & Fornes,
964 2016).
965
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